Plant metabolism: **Enzyme regulation by 14-3-3 proteins**

Paul C. Sehnke and Robert J. Ferl

14-3-3 proteins have been found to regulate the plant enzyme nitrate reductase by reversible phosphoserine binding. Plant plasma-membrane H^+-ATPases, transporters that are activated by the phytotoxin fusicoccin, appear to be regulated in a similar fashion.

Address: Department of Horticultural Sciences, 1253 Fifield Hall, University of Florida, Gainesville, Florida 32611, USA.

Current Biology 1996, Vol 6 No 11:1403–1405

 ISSN 0960-9822

Given their sessile nature, plants need to be particularly responsive to environmental stimuli in order to survive. This environmental adaptation involves signal recognition coupled to the tightly controlled regulation of cellular proteins, such as enzymes and molecular chaperones. Critical enzymatic processes are usually regulated at multiple levels, including, the transcriptional and post-translational levels. Such is the case for nitrate reductase, one of the most regulated of all plant enzymes, which catalyzes a key step in nitrate assimilation — the reduction of absorbed nitrate to nitrite [1]. Recent work has shown that one important mechanism of nitrate reductase regulation involves the binding of a 14-3-3 protein to the phosphorylated enzyme; this appears to be a common mechanism of protein regulation in plants.

Nitrate reductase is a 100 kDa homodimer, each subunit of which is divided into three domains, separated by two hinge regions. The carboxy-terminal electron donor domain, which binds the redox cofactors NADH and FAD, transports electrons *via* an intermediate heme–iron group to the amino-terminal, molybdenum-containing domain, which reduces the bound nitrate to nitrite. The expression of nitrate reductase is inducible and depends on both a light signal and the presence of the substrate nitrate. The enzyme is also subject to post-translational regulation by phosphorylation in response to stimuli such as light levels, the carbon dioxide concentration and nitrate availability.

Nitrate reductase can be inactivated in a matter of hours in response to darkness. This inactivation occurs by a two-step process: phosphorylation of serine 543, within hinge 1 of the protein, followed by binding of nitrate reductase inhibitory protein (NIP) [2–5]. NIP specifically interferes with electron flow between the two metal-dependent cofactor domains of nitrate reductase [5], presumably by altering its conformational state. Removal of part of the amino-terminal domain of nitrate reductase blocks this inactivation process [6]. Nitrate reductase is reactivated upon dephosphorylation by a microcystin-sensitive phosphatase — probably a type 2A phosphatase — and release of NIP [3,4]. Nitrate reductase activity also varies with changes in Mg^{2+} or Ca^{2+} concentration — millimolar or higher levels are needed to allow NIP binding to, and thus inhibition of, phosphorylated nitrate reductase.

Two groups studying the regulation of spinach nitrate reductase have now identified NIP as a member of the highly conserved family of 14-3-3 proteins [7,8]. These dimeric proteins, originally isolated from brain tissue and named for their gel migration pattern, have been identified as components of diverse biomolecular complexes and ascribed multiple functions (reviewed in [9,10]). Both groups were able to produce tryptic fragments of purified NIP that corresponded to conserved sequences from 14-3-3 proteins. Using monoclonal antibodies, Bachmann *et al.* [7] showed that NIP is a 14-3-3 protein immunologically related to the *Arabidopsis* 14-3-3 protein GF14ω. Moorhead *et al.* [8] obtained similar results with polyclonal antibodies to mammalian 14-3-3 proteins.

The precise identification of NIP is complicated by the presence of multiple 14-3-3 isoforms in plants — ten have been identified in *Arabidopsis* already (R.J. Ferl, personal communication). From the work of Moorhead *et al.* [8], it appears that at least four 14-3-3 isoforms are capable of inhibiting nitrate reductase, one of which is highly conserved in *Arabidopsis* (R.J. Ferl, personal communication). Attempts to determine the particular 14-3-3 protein(s) that function as NIP(s) generated multiple sequences representing multiple isoforms. This is not surprising, given that 14-3-3 proteins are able to form both homodimers and heterodimers, but it leaves open the question of the active dimeric state of the molecule. Given the current belief that promiscuous partnering by 14-3-3 proteins increases the potential for signalling by multiple pathways, it is tempting to speculate that different NIPs may lie on distinct pathways transducing the signals from different stimuli inside cells.

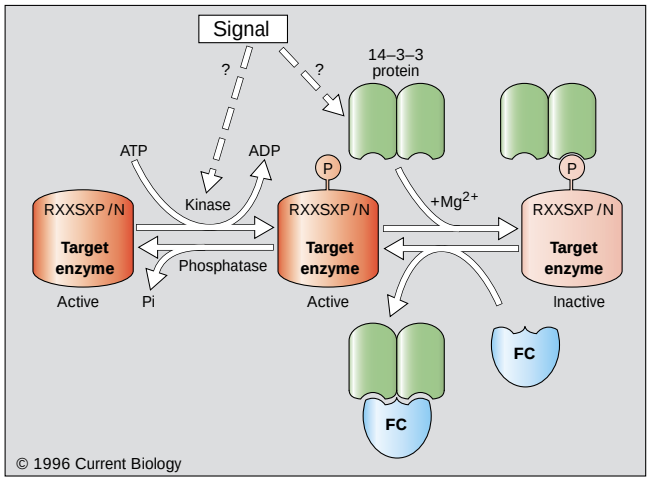
Phosphorylated nitrate reductase is inhibited *in vitro* by recombinant plant (GF14ω), yeast (BMH1, BMH2) and mammalian (ζ,τ) 14-3-3 proteins, as well as a mixture of mammalian brain 14-3-3 proteins [7,8]. Interestingly, all but the recombinant mammalian 14-3-3 proteins are as potent inhibitors as NIP itself. These results with recombinant proteins suggest that the 14-3-3 proteins do not have to be post-translationally modified to have inhibitory activity; they also suggest that heterodimer formation is not necessary for inhibition, as the recombinant proteins

are homodimers [11]. The recombinant proteins, like spinach NIP, all required millimolar levels of Mg^{2+} for inhibition activity. This may be explained by the finding that GF14ω has a low-affinity divalent-cation-binding site near its carboxyl terminus; cation binding alters the structure of this domain, as shown by a change in protease sensitivity, and it is possible that only the Mg^{2+}-bound conformation of the 14-3-3 protein can bind and inhibit phosphorylated nitrate reductase.

Mammalian 14-3-3 proteins have been shown to bind signalling proteins such as the kinase Raf-1, the phosphatase Cdc25 and polyomavirus middle T antigen [9,10,12]. From their binding properties, it has been suggested that 14-3-3 proteins regulate signalling pathways and cell growth [13], but there is little direct evidence for this and the regulatory mechanisms are not known. Studies of the interaction with Raf-1 have indicated that 14-3-3 proteins are phosphoserine-binding proteins [14]. A phosphoserine-containing motif — RXXpSXP/N, where X can be any amino acid — is present in all the proteins found to interact with 14-3-3 proteins, and binding of 14-3-3 proteins to phosphorylated Raf-1 can block dephosphorylation by the phosphatase PP1. The sequence around serine 543 of nitrate reductase is strikingly similar to the phosphoserine 14-3-3-binding site of Raf-1, and Moorhead *et al.* [8] showed that a phosphopeptide based on this Raf-1 sequence can block NIP. These results strongly suggest that mammalian and plant 14-3-3 proteins bind their target proteins in a similar manner, though it appears that Raf-1 binding by 14-3-3 proteins does not require Mg^{2+} [9,10].

Another known association of 14-3-3 proteins is with the fungal phytotoxin, fusicoccin [15]. Fusicoccin causes wilt in almost all higher plants, because it stimulates stomatal opening and thus water loss. This phytohormone-like effect, and other associated physiological effects of fusicoccin, have been attributed to the activation of plasma membrane ion pumps, specifically plasma-membrane H^+-ATPase [16,17]. Moorhead *et al.* [8] showed that fusicoccin can disrupt binding of 14-3-3 proteins to phosphorylated nitrate reductase, suggesting there is competition for binding domains on the 14-3-3 proteins. These findings, and the report that the plasma-membrane H^+-ATPase is regulated by phosphorylation [18], suggest a possible mechanism of H^+-ATPase activation by fusicoccin: fusicoccin might compete for, and remove, inhibitory 14-3-3 proteins from plasma-membrane H^+-ATPase molecules, which would be reactivated as a result. To test this notion, Moorhead *et al.* [8] added the Raf-1-derived phosphopeptide to radish plasma-membrane preparations, and found that it resulted in a three-fold activation of the H^+-ATPase. Identical results were obtained by adding fusicoccin or protein phosphatase 2A, suggesting that phosphorylation allows binding of 14-3-3 proteins to the plasma membrane H^+-ATPase. It should be noted, however, that the plasma-membrane H^+-ATPase lacks a serine in a similar context to the Raf-1 phosphorylation site.

Figure 1



A two-step model for enzyme regulation by 14-3-3 proteins. In the first step, the target protein is phosphorylated by a kinase at a serine residue in the context of a short target sequence; this allows the second step, binding by the 14-3-3 protein that inhibits the target enzyme. The enzyme can be reactivated by competitive removal of the 14-3-3 protein, for example by fusicoccin (FC) in the recent experiments of Moorhead *et al.* [8], or dephosphorylation of the target enzyme by a phosphatase.

Taken together, the nitrate reductase and H^+-ATPase studies point to a common mechanism for enzyme regulation by 14-3-3 proteins (Fig. 1). The target enzyme would be regulated by phosphorylation and binding of a 14-3-3 protein; this process would be contingent upon the cellular levels of the 14-3-3 protein, the kinase and phosphatase that act on the target enzyme, and Mg^{2+}. This complex contingency, as well as the large number of possible 14-3-3 isoform combinations, allows for multiple regulatory controls on the target enzyme activity. Such a scheme may also apply to mammalian systems; indeed, it is interesting to note that most mammalian 14-3-3-binding proteins identified to date are kinases or phosphatases, and may actually be recruited as tools for modification of target enzymes, rather than final targets. This view is consistent with the finding that binding to 14-3-3 proteins does not independently affect the enzymatic activity of the Raf-1 kinase and Cdc25 phosphatase [9,10,12].

Acknowledgements

The authors' work on 14-3-3 proteins is supported by grants from the National Institutes of Health and the USDA competitive grants program. This is journal series number R-05362 of Florida Agricultural Station.

References

1. Campbell WH: Nitrate reductase bichemistry comes of age. *Plant Physiol* 1996, 111:355–361
2. Spill D, Kaiser WM: Partial purification of two proteins (100 kDa and 67 kDa) cooperating in the ATP-dependent inactivation of spinach leaf nitrate reductase. *Planta* 1994, 192:183–188.

3. Douglas P, Morrice N, MacKintosh C: Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (Spinacea oleracea) leaves. *FEBS Lett* 1995, 377:58–65.
4. Su W, Huber SC, Crawford NM: Identification ***in vitro*** of a post-translational regulatory site in the hinge 1 region of ***Arabidopsis*** nitrate reductase. *Plant Cell* 1996, 8:519–527.
5. Bachmann M, Shiraishi N, Campbell WH, Yoo B-C, Harmon AC, Huber SC: Identification of ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. *Plant Cell* 1996, 8:505–517.
6. Nussaume L, Vincentz M, Meyer C, Boutin J-P, Caboche M: Posttranscriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. *Plant Cell* 1996, 7:611–621.
7. Bachmann M, Huber JL, Liao PC, Gage DA, Huber SC: The inhibitor protein of phosphorylated nitrate reductase from spinach (***Spinacia-oleracea***) leaves is a 14-3-3-protein. *FEBS Lett* 1996, 387:127–131.
8. Moorhead G, Douglas P, Morrice N, Scarabel M, Aitken A, MacKintosh C: Phoshorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. *Curr Biol* 1996, 6:1104–1113.
9. Ferl RJ: 14-3-3 proteins and signal-transduction. *Annu Rev Plant Physiol Plant Mol Biol* 1996, 47:49–73.
10. Aitken A: 14-3-3 proteins on the MAP. *Trends Biochem Sci* 1995, 20:95–97.
11. Lu G, Sehnke PC, Ferl RJ: Phosphorylation and calcium binding properties of an Arabidopsis GF14 brain protein homolog. *Plant Cell* 1994, 6:501–510.
12. Conklin DS, Galaktionov K, Beach D: 14-3-3 proteins associate with cdc25 phosphatases. *Proc Natl Acad Sci USA* 1995, 92:10142–10146.
13. Morrison D: 14-3-3: modulators of signaling proteins? *Science* 1994, 266:56–57.
14. Muslin AJ, Tanner JW, Allen PM, Shaw AS: Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 1996, 84:889–897.
15. Korthout HAAJ, de Boer AH: A fusicoccin binding protein belongs to the family of 14-3-3 brain protein homologs. *Plant Cell* 1994, 6:1681–1692.
16. Johansson F, Sommarin M, Larsson C: Fusiococcin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. *Plant Cell* 1993, 5:321–327.
17. De Michelis MI, Rasi-Caldogno F, Pugliarello MC, Olivari C: Fusicoccin binding to its plasma membrane receptor and the activation of the plasma membrane H^+-ATPase III. Is there a direct interaction between the fusicoccin receptor and the plasma membrane H^+-ATPase? *Plant Physiol* 1996, 110:957–964.
18. Xing Ti, Higgins VJ, Blumwald E: Regulation of plant defense response to fungal pathogens: Two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+-ATPase. *Plant Cell* 1996, 8:555–564.